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(54) Title: METHODS FOR PREPARING LIPIDS/POLYNUCLEOTIDE TRANSFECTION COMPLEXES

(57) Abstract

Methods are provided for the preparation of transfection complexes of polynucleotides and cationic lipids suitable for delivering polynucleotides to cells. In particular, polynucleotide/cationic lipid transfection complexes are prepared in the absence of detergent by methods in which polynucleotides and cationic lipids are solubilized in an aqueous solution in the presence of co-solvent, and the removal of co-solvent under conditions which allow polynucleotide and cationic lipid to assemble into transfection complexes. The process produces a substantially homogenous population of tranfection complexes and is scaleable.

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METHODS FOR PREPARING LIPID/POLYNUCLEOTIDE TRANSFECTION COMPLEXES

Field of the Invention

This invention relates to preparation of polynucleotide transfection complexes and their use in delivering polynucleotides to cellshelper lipids, used in conjunction with cationic lipids, for the preparation of liposomes and other lipid-containing carriers of nucleic acids and other substances, for delivery to cells. In particular, the invention relates to methods for preparing complexes of polynucleotides and cationic lipids suitable for transfecting eukaryotic cells *in vivo* and *in vitro*.

Background of the Invention

A number of methods exist for introducing exogenous genetic material to cells, which methods have been used for a wide variety of applications including, for example, research uses to study gene function, and ex vivo or *in vivo* genetic modification for therapeutic purposes. Ex vivo genetic modification involves the removal of specific cells from an animal, including humans, introduction of the exogenous genetic material, and then re-introduction of the genetically modified cells into the animal. By contrast, *in vivo* genetic modification involves the introduction of genetic material directly to the animal, including humans, using an appropriate delivery vehicle, where it is taken up directly by the target cells.

Generally, the various methods used to introduce nucleic acids into cells have as a goal the efficient uptake and expression of foreign genes. In particular, the delivery of exogenous nucleic acids in humans and/or various commercially important animals will ultimately permit the prevention, amelioration and cure of many important diseases and the development of animals with commercially important characteristics. The exogenous genetic material, either DNA or RNA, may provide a functional gene which, when expressed, produces a protein lacking in the cell or produced in insufficient amounts, or may provide an antisense DNA or RNA or ribozyme to interfere with a cellular function in, e.g., a virus-infected cell or a cancer cell, thereby providing an effective therapeutic for a disease state.

Cationic lipids have been developed that greatly facilitate nucleic acid delivery to cells, both *in vitro* and *in vivo*. See, for example, U.S. Patent No. 5,264,618, which describes techniques for using lipid carriers, including the preparation of liposomes and pharmaceutical compositions and the use of such compositions in clinical situations. A number of cationic lipids have been developed, which are generally amphipathic molecules comprising a positively charged headgroup, varying from single to multiple positive charges, linked to hydrophobic lipid tail groups or steroidal groups.

For cationic lipid-mediated delivery, the cationic lipids typically are mixed with a non-cationic lipid, usually a neutral lipid, and allowed to form stable liposomes, which liposomes are then mixed with the nucleic acid to be delivered. The liposomes may be large unilamellar vesicles (LUVs), multilamellar vesicles (MLVs) or small unilamellar vesicles (SUVs). The liposomes are mixed with nucleic acid in solution, at concentrations and ratios optimized for the target cells to be transfected, to form cationic lipid-nucleic acid transfection complexes. Alterations in the lipid formulation allow preferential delivery of nucleic acids to particular tissues *in vivo*. See PCT patent application numbers WO 96/40962 and WO 96/40963.

Nucleic acids are generally large polyanionic molecules which, therefore, bind cationic lipids and other positively-charged carriers through charge interactions. It is believed that the positively charged carriers such as cationic lipids form tight complexes with the nucleic acid, thereby condensing it and protecting it from nuclease degradation. In addition, cationic lipid carriers may act to mediate transfection by improving association with negatively-charged cellular membranes by giving the complexes a positive charge, and/or enhancing transport from the cytoplasm to the nucleus where DNA may be transcribed.

With respect to any of the cationic lipid carriers, transfection efficiency is highly dependent on the characteristics of the cationic lipid/nucleic acid complex. The nature of the complex that yields optimal transfection efficiency depends upon the mode of delivery, e.g. ex vivo or *in vivo*; for *in vivo* delivery, the route of administration, e.g., intravenous, intraperitoneal, inhalation, etc.; the target cell type, etc. Depending on the use, therefore, different carriers will be preferred. In addition to the choice of cationic lipid carrier, transfection efficiency will depend on certain physical characteristics of the complexes as well, such as charge and size. In addition,

the stability of the complexes during storage will be highly dependent on the physical nature of the complexes.

These characteristics depend largely on the method by which the complexes are prepared. Particularly for human therapeutic purposes, therefore, it is desirable to have a method of forming the nucleic acid/polycationic carrier complexes in a highly controllable manner. Further, it is desirable to have a process for preparing the complexes which is highly reproducible and scaleable.

The present invention provides these and related advantages as well.

Relevant Literature

Cationic lipid carriers have been shown to mediate intracellular delivery of plasmid DNA (Felgner et al., (1987) Proc. Natl. Acad. Sci. (USA), 84:7413-7416); mRNA (Malone et al., (1989) Proc. Natl. Acad. Sci. (USA) 86:6077-6081); and purified transcription factors (Debs et al., (1990) J. Biol. Chem. 265:10189-10192), in functional form. Literature describing the use of lipids as carriers for DNA include the following: Zhu et al., (1993) Science, 261:209-211; Vigneron et al., (1996) Proc. Natl. Acad. Sci. USA, 93:9682-9686; Hofland et al., (1996) Proc. Natl. Acad. Sci. USA, 93:7305-7309; Alton et al., (1993) Nat. Genet. 5:135-142; von der Leyen et al., (1995) Proc. Natl. Acad. Sci. (USA), 92:1137-1141; See also Stribling et al., (1992) Proc. Natl. Acad. Sci (USA) 89:11277-11281, which reports the use of lipids as carriers for aerosol gene delivery to the lungs of mice. For a review of liposomes in gene therapy, see Lasic and Templeton, (1996) Adv. Drug Deliv. Rev. 20:221-266.

The role of helper lipids in cationic lipid-mediated gene delivery is described in Felgner et al., (1994) J. Biol. Chem. 269(4): 2550-2561 (describing improved transfection using DOPE); and Hui et al., (1996) Biophys. J. 71: 590-599. The effect of cholesterol on liposomes *in vivo* is described in Semple et al., (1996) Biochem. 35(8): 2521-2525.

A method of preparing cationic lipid/nucleic acid transfection complexes by first forming lipid micelles in the presence of detergent is described in WO 96/37194. The effect of surfactants on DNA/lipid complexes and transfection activity are described in Liu et al., (1996) Pharm. Res. 13(11):1642-1646. The effect of liposome preparation and complex size on cationic lipid-mediated gene delivery is described in Templeton et al., (1997) Nat. Biotech. 15(7):647-652. The use of polyethylene glycol

in DNA/lipid complexes for aerosol delivery is described in Eastman et. al., (1997) Hum. Gene Ther. 8(6):765-773.

Summary of the Invention

The invention provides a method of preparing a cationic lipid/polynucleotide transfection complex, the method comprising co-solubilizing a cationic lipid carrier and a polynucleotide in an aqueous solution in the presence of a co-solvent, then removing the co-solvent such that the polynucleotide and cationic lipid carrier molecules "nucleate" in solution, forming an aqueous dispersion of cationic lipid/polynucleotide transfection complexes. The polynucleotide, cationic lipid and, optionally, non-cationic lipid, are co-solubilized in the ratios desired in the final transfection complex, which ratios will be dependent upon the lipids used, the target cell type and, if administered *in vivo*, the route of administration.

The polynucleotide is typically plasmid DNA, generally including a recombinant expression construct, the DNA encoding a transcription product and operatively linked regulatory elements, whereby the DNA is capable of transcription in the target cells. As used herein, the term "transcription product" is intended to encompass an RNA product resulting from transcription of a nucleic acid sequence, and includes RNA sequences that are not translated into protein (such as antisense RNA or ribozymes) as well as RNAs that are subsequently translated into polypeptides or proteins. Also included is the preparation of complexes including polycationic carriers and oligonucleotides.

In preferred embodiments, the cationic lipid is DOTIM, the neutral lipid is cholesterol, and the lipid and DNA are co-solubilized in a solution of about 70% ethanol and 10% ethyl acetate. Preferably, the co-solvents are removed by dialysis, and the resulting cationic lipid/polynucleotide transfection complexes have an average size of less than about 500 nm.

Specific preferred embodiments of the present invention will become evident from the following more detailed description of certain preferred embodiments and the claims.

Brief Description of the Drawings

Figure 1 shows the density gradient profiles of DNA:cationic lipid complexes. Profiles are measured by flow-cell UV spectrophotometer at 237nm, which is the absorbance of DOTIM. The contents of the centrifuged samples (approx. 13ml) are pumped through the flow-cell at a rate of 1 ml/min. The ordinate represents the approximate location within the centrifuge tube. From top to bottom Figure 1 shows the density gradient profiles of complex preparations: a) prepared by the standard mixing method in a 1:5 DNA/cationic lipid ratio (μg DNA:nmoles cationic lipid); b) prepared by the solution nucleation method in a 1:1 DNA/cationic lipid ratio; and c) prepared by the standard mixing method in a 1:1 DNA/cationic lipid ratio.

Description of Specific Embodiments

The physical nature of polynucleotide/cationic lipid transfection complexes is highly dependent on the method in which they are prepared. The physical nature of the transfection complexes, in turn, is important for the ability of the complexes to transfect target cells. In addition, the stability of the complexes during storage is highly dependent upon their physical nature. Typically, more homogenous compositions are more stable during storage than heterogeneous compositions. Also, compositions containing a higher proportion of active particles utilize starting materials more efficiently, and, in many cases less material will be necessary to achieve desired transfection rates, thereby decreasing any undesirable toxicities associated with excess material.

Typically, transfection complexes are prepared by adding one solution to the other, i.e. nucleic acid to pre-formed liposomes or liposomes to nucleic acid, with constant stirring. Transfection complexes prepared by prior art methods of adding one solution to the other results in a heterogeneous mix of complexes because the environment under which the complexes are formed is constantly changing. In addition, most prior art methods of complex preparation involve first preparing the lipids in a dispersed liposomal form. Thus, most cationic lipid carriers are formulated with a neutral lipid to aid in forming stable liposomal intermediates. The lipids are usually dried as a film in an organic solution such as chloroform, then dispersed in an aqueous solution. In an aqueous environment, the lipids spontaneously form liposomes. The liposomes are formed as a heterogeneous mix unilamellar and multilamellar vesicles, in a range of sizes. They are then usually sonicated or

extruded through membranes with specified pore sizes, usually to form small unilamellar vesicles (SUVs). See, e.g., *Liposome Technology* (CFC Press, NY 1984); *Liposomes* by Ortro (Marcel Schher, 1987); *Methods Biochem Anal.* 33:337-462 (1988). By contrast, the method of the present invention avoids the preparation and use of liposomal intermediates.

The method of the present invention allows the formation of nucleic acid/cationic lipid transfection complexes by a process termed "solution nucleation." In short, the process involves the co-solubilization of the desired components in the ratios desired in the final transfection complex. The solution will contain an aqueous component and one or more co-solvents. Upon co-solubilization, the lipid and polynucleotide components are substantially miscible within the solution. The co-solvent(s) may then be removed, resulting in a substantially aqueous environment. The loss of hydration within the co-solvent(s) may cause the polynucleotides to compact or condense more readily than within a more aqueous environment.

Upon co-solvent removal, individual components will seek a more favorable physical state by assembling into lipid/polynucleotide complexes. They will form complexes by physical interactions such as electrostatic interactions between the cationic lipid and the polynucleotide, hydrophobic interactions between the lipid components, van der Waals forces, etc. Each point where the components assemble into complexes is essentially a "nucleation" event. The method results in all complexes being formed in essentially identical environments, where each component "sees" the other components in the same ratios under the same conditions.

"Transfection" as used herein means the delivery of exogenous nucleic acid molecules to a cell, either *in vivo* or *in vitro*, whereby the nucleic acid is taken up by the cell and is functional within the cell. A cell that has taken up the exogenous nucleic acid is referred to as a "host cell", "target cell" or "transfected cell." A nucleic acid is functional within a host cell when it is capable of functioning as intended. Usually, the exogenous nucleic acid will comprise an expression cassette which includes DNA coding for a gene of interest, with appropriate regulatory elements, which will have the intended function if the DNA is transcribed and translated, thereby causing the host cell to produce the peptide or protein encoded therein. DNA may encode a protein lacking in the transfected cell, or produced in insufficient quantity or less active form, or secreted, where it may have an effect on cells other

than the transfected cell. Other examples of exogenous nucleic acid to be delivered include, e.g., antisense oligonucleotides, mRNA ribozymes, or DNA encoding antisense RNA or ribozymes. Nucleic acids of interest also include DNA coding for a cellular factor which, when expressed, activates the expression of an endogenous gene.

"Transfection efficiency" refers to the relative number of cells of the total within a cell population that are transfected and/or to the level of expression obtained in the transfected cells. It will be understood by those of skill in the art that, by use of appropriate regulatory control elements such as promoters, enhancers and the like, the level of gene expression in a host cell can be modulated. The transfection efficiency necessary or desirable for a given purpose will depend on the purpose, for example the disease indication for which treatment is intended, and on the level of gene expression obtained in the transfected cells.

"Polycation" refers to any molecular entity having multiple positive charges. Because of their positive charges, polycations interact electrostatically with negatively charged polynucleotides, usually condensing the polynucleotide molecules. "Polycationic carrier" refers to a polycation which, when combined with a polynucleotide, forms a complex suitable for transfecting eukaryotic cells. Cationic lipids have been shown to be efficient polycationic carriers for nucleic acid delivery to cells. Typically, cationic lipid carriers are formulated with both cationic and non-cationic lipid (usually neutral lipid) components. Thus, a "lipid carrier" or "cationic lipid carrier" refers to a lipid composition of one or more cationic lipids and, optionally, one or more non-cationic lipids for delivering agents to cells. A lipid carrier may be complexed with other polycations or additional transfection facilitating agents.

"Transfection complex" or "polynucleotide transfection complex" refers to a combination of a polycationic carrier and a nucleic acid, in any physical form, for use in transfecting eukaryotic cells. A transfection complex may include additional moieties, e.g., targeting molecules such as receptor ligands or antibody fragments, or other accessory molecules. For example, nuclear localizing peptides may be included for facilitating transport of the polynucleotide to the cell nucleus. Kalderon et al., (1984) Cell 39:499-509; Chelsky et al., (1989) Mol. Cell Biol. 9:2487-2492; Dingwall & Laskey (1991) Trends Biochem. Sci. 16:478-481. Proteins or peptides may be

included in the transfection complex to facilitate release of the transfection complex from the endosome after internalization. Raja-Walia et al., (1995) Hum. Gene Therap. 2:521-530; Bai et al., (1993) J. Virol. 67:5198-5205. In addition, enzymes involved in transcription and/or translation may be included to facilitate gene expression in the cell cytoplasm without transport to the cell nucleus. Gao & Huang (1993) Nucl Acids Res. 21:2867-2872.

The transfection complexes may also be prepared to include a targeting moiety, to specifically deliver complexes to the desired target cell *in vivo*. Thus, strategies are known in the art for including receptor ligands for delivery to cells expressing the appropriate receptor, or using antibodies or antibody fragments to target transfection complexes to cells expressing a specific cell surface molecule. See WO 96/37194; Ferkol et al., (1993) J. Clin. Invest. 92:2394-2400.

The term "cationic lipid" is intended to encompass lipids that are positively charged at physiological pH, and more particularly, constituitively positively charged lipids comprising, for example, a quarternary ammonium salt moiety. Cationic lipids used for gene delivery typically consist of a hydrophilic polar head group and lipophilic aliphatic chains. Alternatively, cholesterol derivatives having a cationic polar head group are used in a similar manner. Farhood et al., (1992) Biochim. Biophys. Acta 1111:239-246; Vigneron et al., (1996) Proc. Natl. Acad. Sci. (USA) 93:9682-9686.

Cationic lipids of interest include, for example, imidazolinium derivatives (WO 95/14380), guanidine derivatives (WO 95/14381), phosphatidyl choline derivatives (WO 95/35301), and piperazine derivatives (WO 95/14651). Examples of cationic lipids that may be used in the present invention include DOTIM (also called BODAI) (Solodin et al., (1995) Biochem. 34: 13537-13544), DDAB (Rose et al., (1991) BioTechniques 10(4):520-525), DOTMA (U.S. Patent No. 5,550,289), DOTAP (Eibl and Wooley (1979) Biophys. Chem. 10:261-271), DMRIE (Felgner et al., (1994) J. Biol. Chem. 269(4): 2550-2561), EDMPC (commercially available from Avanti Polar Lipids, Alabaster, Alabama), DC-Chol (Gau and Huang (1991) Biochem. Biophys. Res. Comm. 179:280-285), (Behr et al., (1989) Proc. Natl. Acad. Sci. USA, 86:6982-6986), MBOP (also called MeBOP) (WO 95/14651), and those described in WO 97/00241.

Particularly preferred are EDMPC for aerosolized delivery to airway epithelial cells, and DOTIM, DOTAP or MBOP for intravenous delivery to vascular endothelial cells of various organs, particularly the lung. In addition, cationic lipid carriers having more than one cationic lipid species may be used to produce complexes according to the method of the present invention.

Non-cationic lipids of use in transfection complexes are known, and include, for example, dioleoyl phosphatidylethanolamine (DOPE), Hui et al., (1996) Biophys. J. (71):590-599; cholesterol, Liu et al., (1997) Nat. Biotech. (15):167-173; and dilauroyl phosphatidylethanolamine (DLPE) (co-pending patent application serial no. 08/832,749). Normally, cationic lipid and non-cationic lipids are used in approximately equimolar amounts, but need not be.

Additional polycationic carriers include positively charged peptides and proteins, both naturally occurring and synthetic, as well as polyamines, carbohydrates or synthetic polycationic polymers. Examples include polylysine, polyarginine, protamine, polybrene, histone, cationic dendrimer, and synthetic polypeptides based on viral peptides, e.g., having cell binding, endosomal release or nuclear localizing functions, etc. For certain applications, polycationic carriers may include cationic lipid as well as peptide moieties. See, e.g., WO 96/22765.

The nucleic acid may be in any physical form, e.g., linear, circular or supercoiled; single-stranded, double-, triple-, or quadruple-stranded; and further including those having naturally occurring nitrogenous bases and phosphodiester linkages as well as non-naturally occurring bases and linkages, e.g. for stabilization purposes. Preferably it is in the form of supercoiled plasmid DNA. Plasmid DNA is conveniently used for DNA transfections since there are no size constraints on the DNA sequences that may be included, and it can be produced in large quantity by growing and purifying it from bacterial cells.

Polynucleotide transfection complexes are formed by the electrostatic binding between the polynucleotide and the polycationic carrier. In addition to the mixing conditions, the physical structure of such complexes depends on the polycationic carrier and nucleic acid components, the ratios between them, concentrations of each, buffer ionic strength, and the like. Smith et al., (1997) Adv. Drug Deliv. Rev. 26:135-150.

Initially, the components of the transfection complex must be co-solubilized in a solution in the ratios desired in the final complex. The solution will contain water and one or more co-solvents. As used herein, a co-solvent is any solvent other than water. Typically, co-solvents will include polar organic solvents. The solution should be prepared in the absence of salts, which would decrease the solubility of the lipid and polynucleotide components and interfere with the complexation process.

The co-solvent is usually one that is miscible with water and, when combined with the aqueous component, the polynucleotide and lipids are both soluble. The selection of co-solvent will depend on the lipid(s) used. Solubility of many lipids are known or can be determined from, for example, CRC Handbook of Chemistry and Physics, (76th edition, CRC Press, New York, David R. Lide editor), Merck Index (Merck & Co., Inc., Whitehouse Station, New Jersey, 1996) or from the catalogue of lipid vendors such as Avanti Polar Lipids (Birmingham, Alabama). If more than one lipid component is used, for example a cationic lipid and a neutral lipid, the least soluble lipid is the preferred starting point for selecting a co-solvent or combination of co-solvents. Once the solubility of the individual components is determined, the choice of co-solvents and relative amounts may be determined empirically.

Where the resulting transfection complexes are to be administered *in vivo* to humans, the solvents should be selected to minimize any safety or regulatory issues. Short chain alcohols are useful co-solvents because nucleic acids remain soluble up to high levels. A preferred alcohol is ethanol. Other useful co-solvents include chloroform, methanol, propanol, butanol (e.g. *t*-butanol), pentanol, ethyl acetate, ether, pyridine, benzene, polyethylene glycol (MW 1000 to 20,000), dimethyl sulfoxide (DMSO), carbon tetrachloride, phenol, benzene, methylene chloride, acetonitrile, and miscible polymers such as polyvinylalcohol and polyvinylpyrrolidone Preferably, the components are co-solubilized in a solution containing at least about 50% ethanol and less than about 20% ethyl acetate, most preferably, the solution contains about 70% ethanol and about 10% ethyl acetate.

The components may be mixed in any order that avoids precipitation, although the mixing process described in the examples that follow is preferred. The solution will be prepared in the absence of detergent. In a detergent solution, the lipid component will tend to form micelles. It is desirable to simplify the process to avoid issues relating to detergent removal.

Once the transfection components are co-solubilized into a single solution, the co-solvent is then carefully removed. During removal of the co-solvent, the least soluble component of the mixture will begin to come out of solution, causing a "nucleation" event. Upon continued removal of the co-solvent, lipid/polynucleotide complexes will form around such "nucleation" sites, resulting in an aqueous solution comprising substantially homogeneous lipid/polynucleotide transfection complexes.

The co-solvent is preferably removed by dialysis. Other means of co-solvent removal include, for example, diafiltration, tangential flow filtration, dilution, heating, freezing, etc. When removed by dialysis, any dialysis membrane may be used that is compatible with the solvent system used. A wide variety of dialysis membranes are available commercially, and solvent compatibility is generally available from the manufacturers' specifications. Preferably the dialysis membrane has the smallest molecular weight cutoff available. As an example, with an ethanol, ethyl acetate co-solvent system, a regenerated cellulose membrane with a molecular weight cutoff of about 12,000 to 14,000 daltons may be used, such as the Spectra/Por #2 dialysis membrane (Spectrum, Houston, Texas).

Dialysis will be performed against an aqueous solution. The dialysis buffer may be any buffer suitable for the subsequent uses of the complexes, and may include any physiologically acceptable buffer or no buffer. If the complexes will not be used immediately, but will be stored before use, the dialysis buffer selected will depend primarily on the lipid components of the complexes and will be of a composition and pH designed to preserve the stability of the complexes. Preferably, the dialysis buffer is a low ionic strength buffer to minimize interference by any additional ions in the complexation process. A low-ionic strength solution means a solution having a conductivity less than about 35 mS, preferably less than about 10 mS, and most preferably less than about 1 mS. Desirably, the dialysis solution will contain no salts. Where the resulting complexes will be used directly *in vivo*, a preferred dialysis buffer is 5% dextrose in 5 mM Tris-HCl (pH 8.0). Dialysis should be performed against a large excess of dialysis buffer, e.g., at least about 500-fold, and may be 1000-fold or greater.

If desired, the complexes may be concentrated after dialysis. The degree of concentration will depend on the desired use of the complexes, for example, any limitations in volume due to the intended route of administration. Methods for

concentration include, for example, vacuum dialysis, centrifugation, lyophilization, evaporation, and tangential flow filtration.

A number of analytical methods are known for characterizing the complexes prepared according to the method of the invention. Visual inspection may provide initial information as to aggregation of the complexes. Spectrophotometric analysis may be used to measure the optical density, giving information as to the aggregated state of the complexes; surface charge may be determined by measuring zeta potential; agarose gel electrophoresis may be utilized to examine the amounts and physical condition of the polynucleotide molecules in the complexes; particle sizing may be performed using commercially available instruments; HPLC analysis will give additional information as to resulting component ratios and any component degradation; and dextrose or sucrose gradients may be used to analyze the composition and heterogeneity of complexes formed.

The preferred size of the resulting complexes will depend on the desire use. For intravenous administration, the size is preferably less than about five microns, more preferably less than about 500 nm. For aerosol administration, the size should be less than about 500 nm, preferably around 100 nm or less. The size of the resulting complexes may be altered by adjusting the pH, the lipid:polynucleotide concentrations or ratios, the ratios of the lipid components, or by adjusting physical parameters such as temperature, viscosity or agitation as known for other nucleation processes. See, e.g, Mullin, J.W., Crystallization, 3rd Ed. (Butterworth-Heinemann, Oxford, 1993)

It will be appreciated that using the method of complex preparation described herein, polynucleotide transfection complexes may be prepared in a variety of formulations depending of the desired use. Uses contemplated for the complexes of the invention include both *in vivo* and *in vitro* transfection procedures corresponding to those presently known that use cationic lipid carriers, including those using commercial cationic lipid preparations, such as Lipofectin(, and various other published techniques using conventional cationic lipid technology and methods. See, generally, Lasic and Templeton (1996) Adv. Drug Deliv. Rev. 20: 221-266 and references cited therein. Thus, the ratios of each component in the complexes, final concentrations, buffer solutions, and the like are easily adjusted by adjusting the starting components. The method allows the resulting transfection complexes to the

prepared in a highly controlled fashion, efficiently using starting materials and yielding active transfection complexes.

Cationic lipid-nucleic acid transfection complexes can be prepared in various formulations depending on the target cells to be transfected. See, e.g., WO 96/40962 and WO 96/40963. While a range of lipid/polynucleotide complex formulations will be effective in cell transfection Since the activity of a given cationic lipid-nucleic acid transfection complex in transfecting cells *in vitro* does not correlate with *in vivo* activity, optimum conditions are determined empirically in the desired experimental system. Lipid carrier compositions may be evaluated by their ability to deliver a reporter gene (e.g. CAT which encodes chloramphenicol acetyltransferase, luciferase, or (-galactosidase) *in vitro*, or *in vivo* to a given tissue in an animal, such as a mouse.

For *in vitro* transfections, the various combinations are tested for their ability to transfect target cells using standard molecular biology techniques to determine DNA uptake, RNA and/or protein production. Typically, *in vitro* cell transfection involves mixing nucleic acid and lipid, in cell culture media, and allowing the lipid-nucleic acid transfection complexes to form for about 10 to 15 minutes at room temperature. The transfection complexes are added to the cells and incubated at 37°C for about four hours. The complex-containing media is removed and replaced with fresh media, and the cells incubated for an additional 24 to 48 hours.

In vivo, particular cells can be preferentially transfected by the use of particular cationic lipids for preparation of the lipid carriers, for example, by the use of EDMPC to transfect airway epithelial cells (WO 96/40963) or by altering the cationic lipid-nucleic acid formulation to preferentially transfect the desired cell types (WO 96/40962). Thus, for example, in circumstances where a negatively charged complex is desired, relatively less cationic lipid will be complexed to the nucleic acid resulting in a higher nucleic acid: cationic lipid ratio. Conversely, in circumstances where a positively charged complex is desired, relatively more cationic lipid will be complexed with the nucleic acid, resulting in a lower nucleic acid: cationic lipid ratio.

The lipid mixtures are complexed with DNA in different ratios depending on the target cell type, generally ranging from about 6:1 to 1:20 μg DNA:nmole cationic lipid. For transfection of airway epithelial cells, e.g., via aerosol, intratracheal or intranasal administration, net negatively charged complexes are preferred. Thus, preferred DNA:cationic lipid: DNA ratios are from about 10:1 to about 1:20,

preferably about 3:1. For intravenous administration, preferred DNA:cationic lipid: DNA ratios range from about 1:3 to about 1:20 μg DNA: nmole cationic lipid, most preferably, about 1:6 to about 1:15 μg DNA: nmole cationic lipid. Additional parameters such as nucleic acid concentration, buffer type and concentration, etc., will have an effect on transfection efficiency, and can be optimized by routine experimentation by a person of ordinary skill in the art.

Delivery can be by any means known to persons of skill in the art, e.g., intravenous, intraperitoneal, intratracheal, intranasal, intramuscular, intradermal, etc. PCT patent application WO 96/40962 describes the preparation and use of cationic lipid carriers for *in vivo* DNA delivery. For aerosol administration, via intranasal or intraoral delivery, the cationic lipid-nucleic acid transfection complex will withstand both the forces of nebulization and the environment within the lung airways and be capable of transfecting lung cells. Techniques for delivering genes via aerosol administration of cationic lipid-DNA transfection complexes is described in U.S. Patent No. 5,641,662.

The following examples are offered by way of illustration and not by way of limitation.

EXAMPLES

Example 1: Complex Preparation by Solution Nucleation

Plasmid p4119 containing the CAT reporter gene under the control of the HCMV promoter was prepared at a concentration of 8.2 mg/ml in 10 mM Tris, pH 8.0. 100 mg cholesterol (Sigma, St. Louis, MO) and 100 mg DOTIM (Sigma) were each dissolved into 5 ml ethyl acetate solutions, making 20 mg/ml solutions (final concentrations of 28.75 mM DOTIM and 51.7 mM cholesterol).

The DNA/lipid solution was prepared in a 4 ml glass vial by mixing 1400 μl EtOH, 321 μl water, and 76.2 μl DNA. Then 165.16 μl ethyl acetate was added, followed by addition of 12.1 μl 51.7 mM cholesterol and 21.74 μl 28.75 mM DOTIM. The final solution contained 70% EtOH and 10% ethyl acetate; 3.125 mM DOTIM and 3.125 mM cholesterol for a 1:1 molar ratio; and DNA in a final concentration of 0.3125 mg/ml for a 1:1 DNA:cationic lipid ratio The co-solubilized DNA/lipid solution was dialyzed against 1 liter of 5% dextrose, 10 mM Tris, pH 8.0 for four

hours usinga Spectra/Por#2 dialysis membrane (regenerated cellulose: MW cuoff 12Kd-14 Kd)

DNA/lipid complexes were also prepared by standard mixing methods as follows. Liposomes were prepared by first dissolving the lipids (DOTIM and cholesterol) in a mixture of chloroform and methanol (1:1 molar ratio) and lipid films were formed with a rotary evaporator. The films were hydrated with 5% dextrose in water (D5W) at room temperature and the resulting liposomes extruded through a series of membranes having pore sizes of 400nm, 200nm, and 50nm.

DNA-liposome complexes were prepared at a 1:5 DNA:cationic lipid ratio (mg DNA:umole cationic lipid) by adding the DNA, in a solution at 0.625 mg/ml concentration in D5W to the solution containing liposomes, in an equal volume, with constant stirring, using a Hamilton Dilutor 540B (Hamilton, Reno, Nevada). DNA-liposome complexes were also prepared at a 1:1 DNA:cationic lipid ratio (mg DNA:umole cationic lipid) in a similar manner except that the solution containing liposomes was added to the solution containing the DNA.

Example 2: Characterization of DNA/lipid complexes

DNA/lipid complexes were sized using a NiComp 370 particle sizer and found to be 135 nm (78 in diameter. HPLC analysis was performed using a 10 μl sample, analyzed on a Shimadzu LC-10AD HPLC equipped with an Altima C8 column, 250 cm x 4.6 mm, ID 5 (m, Model #88075. The column was previously equilibrated with a mobile phase consisting of 65% acetonitrile, 25% v/v methanol, 9.9% v/v water, and 0.1% v/v trifluoroacetic acid. Following injection, mobile phase is run at a rate of 1 ml/min, 37(C for 20 minutes. Elution peaks are detected by UV absorbance at 215 nm.

The results showed the final complexes contained 42.3% DOTIM and 42.2% cholesterol, maintaining the original 1:1 molar ratio. By agarose gel electrophoresis, plasmid DNA was visible in its native forms.

Dextrose gradients (5% w/v to 20% w/v) were prepared using the BioComp Gradient Master (BioComp Instruments, Inc., New Brunswick, Canada). At room temperature, centrifuge tubes (14 x 89 mm) were half-filled with 5% dextrose followed by careful addition of 6 ml of 20% dextrose to the bottom of the tube with a syringe and canula. The tubes were placed in the Gradient Master and programmed to produce the linear gradients (time = 2 min 25 sec., angle = 81.5°, speed setting = 15).

The gradients were allowed to equilibrate to 4(C for about 4 hrs. Approximately 300 μl sample was loaded to the top of the gradient, and spun for 2 hrs at 40,000 rpm and 4°C using a Beckman XL-70 ultracentrifuge with a SW-41 rotor. The centrifuged gradients were loaded into a tube piercing apparatus (Brandell) and 50% w/v dextrose was pumped at 30 ml/min into the bottom of the tube. The contents of the tube were forced through an on-line UV/VIS spectrophotometer (Rainin) and absorbance was measured at 237nm (DOTIM absorbance). The data was analyzed using Rainin HPLC Dynamax software run on an Apple Macintosh Quadra 610.

Figure 1 shows the density gradient profile of DNA/lipid complexes prepared according to the methods described above. From top to bottom, Figure 1 shows the density gradient profiles of complex preparations: a) prepared by the standard mixing method in a 1:5 DNA/cationic lipid ratio (μg DNA:nmoles cationic lipid); b) prepared by the solution nucleation method in a 1:1 DNA/cationic lipid ratio; and c) prepared by the standard mixing method in a 1:1 DNA/cationic lipid ratio.. The density gradient profile shows that the solution nucleation method produces a less heterogeneous mixture of DNA-lipid complexes than that prepared by standard mixing methods.

All publications and patent applications cited herein are hereby incorporated by reference to the same extent as if fully set forth herein.

The invention now being fully described, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made thereto without departing from the spirit or scope of the appended claims.

We claim:

1. A method of preparing a polynucleotide/cationic lipid transfection complex, said method comprising:

a) providing an aqueous solution comprising a cationic lipid, a polynucleotide, and a co-solvent, in the absence of a detergent, and

b) removing the co-solvent to form a polynucleotide/cationic lipid transfection complex.

2. The method according to claim 1 wherein the solution further comprises a non-cationic lipid.

3. The method according to claim 1 wherein the co-solvent is a polar organic solvent.

4. The method according to claim 1 wherein at least one co-colvent is selected from the group consisting of ethanol, chloroform, methanol, propanol, butanol, pentanol, ethyl acetate, ether, pyridine, benzene, polyethylene glycol, dimethyl sulfoxide, carbon tetrachloride, phenol, benzene, methylene chloride, acetonitrile, polyvinylalcohol and polyvinylpyrrolidone.

5. The method according to claim 3 wherein the solution comprises at least about 50% short-chain alcohol.

6. The method according to claim 5 wherein the short-chain alcohol is ethanol.

7. The method according to claim 1 wherein the polynucleotide is DNA.

8. The method according to claim 1 wherein the polynucleotide is RNA.

9 The method according to claim 7 wherein the DNA is plasmid DNA.

10. The method according to claim 1 wherein the solution further comprises a synthetic cationic polymer.

11. The method according to claim 1 wherein the solution further comprises a polypeptide.

12. The method according to claim 1 wherein the cationic lipid:polynucleotide ratio in the polynucleotide transfection complex is in the range of from about 10:1 to 1:20 μgram polynucleotide:nmole cationic lipid.

13. The method according to claim 1 wherein the cationic lipid is selected from the group consisting of DOTIM, DDAB, DOTMA, DOTAP, DMRIE, EDMPC, DC-Chol, and MBOP.

14. The method according to claim 2 wherein the non-cationic lipid is selected from the group consisting of DOPE, cholesterol and DLPE.

15. The method according to claim 1 wherein the polynucleotide transfection complex has an average size of less than about five microns.

16. The method according to claim 15 wherein the polynucleotide transfection complex has an average size of less than about 500 nanometers.

17. A polynucleotide transfection complex prepared according to the method of claim 1.

18. A method of delivering exogenous genetic capability to a eukaryotic cell comprising administering an effective amount of the polynucleotide transfection complex of claim 17.

19. The method according to claim 18 wherein said administering is *in vitro*.

20. The method according to claim 18 wherein said administering is *in vivo*.

21. The method according to claim 20 wherein said administering is by a method selected from the group consisting of intravenous, intraperitoneal, intradermal, subcutaneous, intramuscular, intratracheal, inhalation, topical, direct intra-organ injection and direct intratumoral injection.

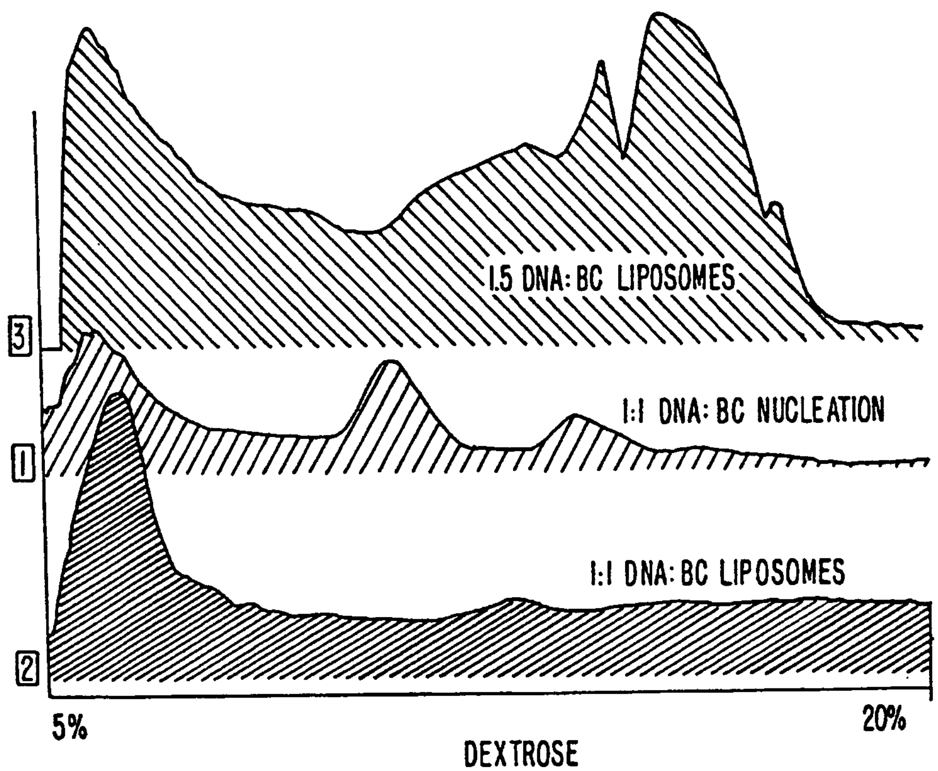
1.5 DNA: BC LIPOSOMES
1:1 DNA: BC NUCLEATION
1:1 DNA: BC LIPOSOMES
3
1
2
5%
20%
DEXTROSE

FIG. 1.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/19936

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :264/4.1, 424/1.21, 417, 450; 514/812; 435/172.3; 514/44,12,2,3
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 264/4.1, 424/1.21, 417, 450; 514/812; 435/172.3; 514/44,12,2,3; 935/54

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, STN, cation?, lipid#, liposome#, aqueous, cosolvent#, co(w)solvent#, chthanol, etoh, methanol, meoh, chloroform, propanol

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 4,897,355 A (EPPSTEIN et al) 30 January 1990, see entire document.	1-21
Y	US 5,334,761 A (GEBEYEHU et al) 02 August 1994, see entire document.	1-21
Y	US 5,451,661 A (WAN) 19 September 1995, see entire document.	1-21
Y	REIMER et al, Formation of novel hydrophobic complexes between cationic lipids and plasmid DNA, Biochemistry, October 1995, Volume 34, pages 12877-12883.	1-21

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "B" earlier document published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed
- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- "&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
15 DECEMBER 1998	25 JAN 1999

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/19936

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	CAMPBELL et al, Lipofection reagents prepared by a simple ethanol injection technique, Chemical Abstracts, July 1995, Volume 123, No. 1, page 524, abstract no. 5027g, see entire document.	1-21
Y	KOIV et al, Influence of calcium and ethanol on the aggregation and thermal phase behavior of L-dihexadecylphosphatidylcholine liposomes, Chemical Abstracts, February 1993, Volume 118, No. 7, pages 327-328, abstract no. 54658u, see entire document.	1-21

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/19936

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

A61K 51/00, 38/00, 9/127, 31/70; A01N 25/26; B01J 13/02; C12N 15/00